Molecular Pharmacology Fast Forward. Published on August 27, 2007 as doi:10.1124/mol.107.039370
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Activation of Nrf2 by Toxic Bile Acids Provokes Adaptive Defense Responses to Enhance Cell Survival at the Emergence of Oxidative Stress

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Molecular Pharmacology Fast Forward. Published on August 27, 2007 as DOI: 10.1124/mol.107.039370
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a) Running Title: Nrf2 counteracts toxic bile acids.

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c) The number of

text pages: (from Introduction to Discussion): 17

tables: 0

figures: 6

references: 38

words

Abstract: 249

Introduction: 587

Discussion: 1017

d) Abbreviations

ARE, antioxidant-responsive element, BSEP, bile-salt export pump; BSO, buthionine sulfoximine; CA, cholic acid, ChIP, chromatin immunoprecipitation; CYP3A, cytochrome P450 3A; CYP7A1, cytochrome P450 7A1; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid, FRL, ferritin light subunit; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GCL, glutamate cysteine ligase; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate cysteine ligase modulatory subunit; GSH, glutathione; GSTA, glutathione s-transferase A; GSTP1, glutathione s-transferase P1; HO1, heme oxygenase 1; NAC, N-acetyl-L-cysteine; NQO1, NAD(P)H quinone oxidoreductase; Nrf2, nuclear factor (erythroid-2 like) factor 2; NTCP, Na(+)-dependent taurocholate cotransporting polypeptide; LCA, lithocholic acid; LDH, lactate dehydrogenase; siRNA, small-interfering RNA; ROS, reactive oxygen species; TRx1, thioredoxin reductase 1; UDCA, ursodeoxycholic acid.

Abstract

Oxidative stress, causing necrotic and apoptotic cell death, is associated with bile acid toxicity. Using liver (HepG2, Hepa1c1c7, and primary human hepatocytes) and intestinal (C2bbe1, a Caco-2 subclone) cells, we demonstrated that toxic bile acids, such as lithocholic acid (LCA) and chenodeoxycholic acid, induced the NF-E2-related factor 2 (Nrf2) target genes, especially glutamate-cysteine ligase subunits (GCLM and GCLC), the rate-limiting enzyme in glutathione (GSH) biosynthesis, and thioredoxin reductase 1. Nrf2 activation and induction of Nrf2 target genes were also evident in-vivo in the liver of CD-1 mice treated 7-8 h or 4 d with LCA. Silencing of Nrf2 via small-interfering RNA suppressed basal and bile acid-induced mRNA levels of the above genes. Consistent with this, overexpression of Nrf2 enhanced, but of dominant-negative Nrf2 attenuated, Nrf2 target gene induction by bile acids. The activation of Nrf2-ARE (antioxidant responsive element) transcription machinery by bile acids was confirmed by increased nuclear accumulation of Nrf2, enhanced ARE-reporter activity, and increased Nrf2 binding to ARE. Importantly, Nrf2 silencing increased cell susceptibility to LCA toxicity, as evidenced by reduced cell viability, and increased necrosis and apoptosis. Concomitant with GCLC/GCLM induction, cellular glutathione (GSH) was significantly increased in bile acid-treated cells. Cotreatment with N-acetyl-L-cysteine, a GSH precursor, ameliorated LCA toxicity, whereas cotreatment with buthionine sulfoximine, a GSH synthesis blocker, exacerbated it. In summary, this study provides molecular evidence linking bile acid toxicity to oxidative stress. Nrf2 is centrally involved in counteracting such oxidative stress by enhancing adaptive antioxidative response, particularly GSH biosynthesis, and hence cell survival.

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Exposure to excessive bile acids is toxic to the cells, contributing an etiopathological factor to a number of liver and intestinal diseases such as cholestasis and colorectal cancer (Debruyne *et al.*, 2002; Rao *et al.*, 2001). Among the bile acids, lithocholic acid (LCA), a hydrophobic secondary bile acid produced by colonic microflora on chenodeoxycholic acid (CDCA), is the most toxic bile acid with genotoxic and mutagenesis-enhancing properties (Kawalek, *et al.*, 1983; Kozoni *et al.*, 2000). In rodents, it induces intrahepatic cholestasis-like hepatotoxicity (Staudinger *et al.*, 2001) and promotes chemical-induced colon carcinogenesis (Kozoni *et al.*, 2000). CDCA, the most hydrophobic primary bile acid, is able to cause severe liver injury in species like rhesus monkey, and mild hepatotoxicity in humans; its chronic administration results in increased colonic production of LCA (Hofmann, 2004).

The integrity and coordination of efficient hepatic bile flow and intestinal bile extraction is hence critical for species survival. The liver and intestinal cells achieve this through a concerted network involving the nuclear transcription factors, such as farnesoid X receptor (FXR), vitamin D receptor (VDR), retinoid X receptor (RXR), liver X receptor (LXR), pregnane X receptor (PXR) and/or constitutive androstane receptor (CAR). These receptors regulate bile-metabolizing and -conjugation enzymes, and bile transporters to prevent excessive accumulation of bile acids (Eloranta & Kullak-Ublick, 2005). Bile acids are regarded as signaling molecules which facilitate synchronization of the above regulators in their handling of cellular bile fate. The crosstalk among these receptors is important in maintaining homeostasis of physiological bile extraction, constituting the baseline protection against bile acid toxicity.

On the other hand, increased cellular production of reactive oxygen species (ROS) and oxidative stress has been implicated in exposure to toxicological concentrations of bile acids. Bile acid-induced oxidative stress results from induction of membrane permeability transition consequent to mitochondrial toxicity and activation of death receptors (CD95), which subsequently lead to apoptosis, via activation of pro-apoptotic effectors caspases, and necrosis (Palmeiro & Rolo, 2004). Whether there exists any regulator to counteract such oxidative stress and progression of bile acid toxicity is presently unknown. Due to its important role as an oxidative stress sensor and anti-

apoptosis factor (Itoh *et al.*, 2004), we hypothesized that the nuclear factor (erythroid 2-like) factor 2 or Nrf2, may play a central role by enhancing adaptive response and cell survival during exposure to excess bile acids.

Nrf2, a basic leucine zipper transcription factor which binds to antioxidant responsive element (ARE), is a chief regulator for many antioxidative, cytoprotective genes (Kensler *et al.*, 2006). Among Nrf2 target genes, the glutamate cysteine ligase (GCL), composed of modulatory (GCLM) and catalytic (GCLC) subunits, is the rate-limiting enzyme for cellular biosynthesis of glutathione (GSH), an important intracellular antioxidant in preserving redox balances. Emerging studies have shown that Nrf2 is a multi-organ protector against various toxic reactive insults; among others are chemical carcinogens (Ramos-Gomez *et al.*, 2001) and acetaminophen (Chan *et al.*, 2001). Hence, robust Nrf2 activation in the cell maybe a critical adaptive response to overcome oxidative stress-induced disease processes. However, Nrf2 activation is not merely a cellular response to all circumstances of oxidative stress as exposure to some oxidative stress inducers such as high dose UVB ray would in turn result in Nrf2 deactivation (Kannan & Jaiswal, 2006). Presently, it is not known if toxic bile acids could activate Nrf2.

In this study, we combined *in vitro* and *in vivo* approaches to demonstrate that Nrf2 is activated by cytotoxic bile acids, thereby inducing genes, such as GCL and hence GSH biosynthesis, to protect the cells against bile acid toxicity.

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Materials and Methods

Cell Culture and Chemicals. The human hepatoma-derived HepG2 (ATCC; Manassas, VA) and mouse hepatoma-derived Hepa1c1c7 (a gift from Dr. Patricia Harper, The Hospital for Sick Children, Toronto, ON) were maintained in α -MEM with 10% fetal bovine serum (FBS). C2bbe1, a subclone of colon carcinoma Caco-2 which displays a more homogeneous brush-border epithelial-like morphology (ATCC), were maintained in DMEM supplemented with 10% FBS, 1.5 g/L sodium bicarbonate and 10 mg/L holo-transferrin. The human primary hepatocytes were purchased from Celprogen (San Pedro, CA) and grown in specially formulated serum-free growth media (Celprogen). Experiments of all secondary cell lines were conducted within 10 cell passages. Treatments were given at ~80% confluence for all cell lines except for C2bbe1. Because C2bbe1 cells differentiate to mature colonocytes at confluence, treatments to this cell line were given 2-3 d post-confluency. All chemicals were purchased from Sigma (St. Louis, MO), unless otherwise indicated. Test bile acids were dissolved in dimethyl sulfoxide (DMSO)(0.2% v/v). Oligonucleotides were synthesized at the Toronto Centre for Applied Genomics or Integrated DNA Technologies (IDT; Coralville, IA).

In-vivo Mouse Experiments. The animal care and experimental procedures were approved by the Animal Care Committee at the University of Toronto and the Hospital for Sick Children. To examine whether Nrf2 target genes may have been modulated during acute exposure to LCA prior to the onset of symptomatic liver injury, 9-12 wk CD-1 mice (Charles River; Montreal, Quebec) were injected i.p. a single dose of LCA at 125 mg/kg body wt. dissolved in sterilized DMSO (final amount <1% body wt.). Mice were killed 7-8 h after the treatment. In a separate experiment aiming to investigate changes in similar genes upon extended treatment with LCA, mice were injected the same dose of LCA dissolved in sterilized corn oil (final amount ~2 % body wt.) twice daily for 4 d. This treatment protocol has been used in the past to induce cholestatic liver injury in mice (Staudinger et al., 2001). Mice were killed 16 h after the last dosing. The use of corn oil as a solvent for LCA in the extended treatment protocol was to avoid the possible toxicity with chronic exposure to DMSO. At necropsy, portions of .their livers were sampled in RNAlater reagent (Invitrogen, Carlsbad, CA) and

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neutral-buffered 10% formalin for mRNA and histological analyses, respectively. Nuclear protein extraction of chilled livers was carried out using Nuclear Extraction Kit (Panomics, Fremont, CA). Sera of mice were collected for analysis of liver function/injury markers: total bilirubbin (TBL), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and γ-glutamyl transferase (GGT) using established automated methods (Dept. of Paediatric Laboratory Medicine, The Hospital for Sick Children, Toronto, ON).

cDNA Synthesis and Quantitative Reverse-Transcription PCR (qRT-PCR). Total RNA was isolated with RNeasy Kit (Qiagen; Valencia, CA) and reverse-transcribed into cDNA using random hexamers and Moloney murine leukemia virus (MMLV) or SuperScript II reverse transcriptase (Invitrogen). Aliquots of cDNA equivalent to 100 ng RNA were used for real-time PCR performed on Applied Biosystems (ABI; Foster City, CA) 7500 Real-Time PCR System or Prism 7700 Sequence Detection system with reaction mode set at 50 ^{0}C (2 min), 95 ^{0}C (20 s), followed by 40 cycles of 95 ^{0}C (15 s) and 56 ^{0}C or 60 ^{0}C (1 min). The primers for ribosomal 18S, β-actin, tata-box binding protein (TBP), GAPDH, GCLM, GCLC and NQO1 were purchased from pre-designed and -optimized Taqman primer-probe sets (Assay-on-demand gene expression probe, ABI), whereas custom-made primers for SYBGreen real-time PCR detection were used for the other gene transcripts (primer sequences available upon request). To assure specificity, primer pairs were designed to span across two neighboring exons and detection of a single peak in dissociation curve analysis. The ΔΔCt method (Livak & Schimittgen, 2001) was employed to quantify the amplification-fold difference between treatment and vehicle-treated control groups, with Ct value of target genes being adjusted to individual housekeeping gene (GAPDH, β-actin, TBP and/or 18S) whichever expression was not affected by treatment protocols. Measurements were done duplicate or triplicate with variability < 0.5 Ct.

Immunoblotting. Whole Cell lysate was prepared in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail (Roche). 10-30 μg protein was dissolved in 4-12% bis-tris gel

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(NuPage® Novex gel system, Invitrogen) and transferred onto a nitrocellulose membrane (Amersham Biosciences; Piscataway, NJ). Primary antibodies (working concentration) used were: rabbit polyclonal anti-GCLC Ab-1 (1:2000) (NeoMarkers; Fremont, CA), rabbit antiserum against GCLM (1:3000) (custom-made; Alpha Diagnostic; San Antonio, TX; see below), rabbit polyclonal anti-Nrf2 c-20 (1:750)(Santa Cruz Biotechnology; Santa Cruz, CA), rabbit polyclonal anti-TRx1 (1:3000)(Abcam; Cambridge, MA), mouse monoclonal anti-β-actin (1:10000) (Sigma) and goat polyclonal anti-lamin B c-20 (1:200) (Santa Cruz). Based on analyses of hydrophilicity, antigenicity, accessibility and sequence homology with other related proteins, an antiserum against a peptide (amino acids 29-45) of human GCLM was raised in rabbits. The immunogenicity and specificity were checked by ELISA, and its ability to detect a ~28 kDa protein (predicted size of GCLM) with reactivity halted after pre-absorption of the antibody in excess immunogen. To ensure equal loading for whole cell lysate and nuclear protein, β-actin and lamin B, respectively, were probed on the same stripped blot membranes after being used for detecting target proteins.

RNA interference (RNAi). A combo of four gene-specific small-interfering RNA (siRNA) against human Nrf2 (NM_006164) was used (Dharmacon SMARTpool® siRNA reagent, Thermo Fisher Scientific, Lafayette, CO; Cat.#: M-003755). Overnight-seeded HepG2 and C2bbE1 cells at ~40% and ~60% confluence, respectively, were transfected for 48 h with 50 nM siRNA against Nrf2 (siNrf2) or equal molar mismatched siRNA controls (siCtr). These siRNAs were earlier complexed with liposome carrier Dharmafect I (Dharmacon) at 0.2 μL/nM siRNA concentration in serum-free Opti-MEM (Invitrogen). Under this condition, the transfected cells after 48 h appeared normal morphologically and did not differ from untransfected cells in cell viability and mRNA levels of inflammatory marker IL-6 (not shown). Treatments with bile acids were then followed for 16-18 h. To ensure achieving functional and specific silencing, the mRNA levels of Nrf2, known Nrf2 target genes and homologous subtypes Nrf1 and Nrf3 were compared between siNrf2 and siCtr groups before and after treatments in all experiments.

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Plasmid Constructs. The expression vectors for Nrf2 (pEF_Nrf2), dominant negative Nrf2 (pEF_DNrf2) and empty vector (pEF) were kindly provided by Dr. Jawed Alam (Ochsner Clinic Foundation, New Orleans, LA). To make an ARE-reporter construct (pGL3_ARE), a DNA duplex (CGGGGTACCGCCCGCACAAAGCGCTGAGTCACGGGGAGGCAGATCTTCC) (core ARE was underlined; -3595/-3625 of hGCLC gene) containing the indispensable ARE motif of GCLC (-3604/-3614)(Mulcahy *et al.*, 1997) with *Kpn 1*/*Bgl II* at 5' and 3' ends, respectively, was constructed by annealing two PAGE-purified complementary oligonucleotides. This insert was ligated to similar restriction enzyme sites of pGL3 luciferase reporter plasmid with SV-40 promoter (Promega; Madison, WI). Similar reporter construct has been successfully used previously (Mulcahy *et al.*, 1997). The responsiveness and robustness of our ARE reporter to Nrf2 transactivation was confirmed by testing of a panel of Nrf2 activators such as tert-butylhydroquinone (BHQ), lipoic acid and diethyl maleate (not shown), as well as cotransfection with Nrf2 and dominant negative Nrf2 expression vectors. To assure specificity, a mutant ARE reporter construct (pGL3_mARE) introducing three point mutations on ARE was cloned by PCR-mediated site-directed mutagenesis using pfu turbo® DNA polymerase (Stratagene) with complementary primers 5'-AGCtaTGAGgCACGGGGAGGCAG-3' (underlined sequence is core ARE; lower cases are mutated nucleotides) on the template pGL3_ARE. The PCR condition was 95 ^{0}C (5 min) followed by 22 cycles of 95 ^{0}C (15 s), 55 ^{0}C (30 s), 68 ^{0}C (10 min), and a final extention of 68 ^{0}C (10 min). The template was then digested by *Dpn 1* and mutant clones were transformed in XL-1 blue competent cells (Stratagene; La Jolla, CA). Successful insertion and mutation introduction were confirmed by sequencing. The cDNA clone of human Na(+)-taurocholate co-transporting polypeptide (NTCP) (Origene Technologies, Rockville, MD) was subcloned into the *Not 1* site of pTarget expression vector (Promega), and stably transfected into HepG2. Stable clones transfected with NTCP or empty vector (pTarget) were selected using 500 µg/mL G-418 growth media.

Transfection, Reporter Assays and Overexpression Studies. HepG2 cells at ~50% confluence were transiently transfected overnight with 0.1 µg of the *firefly* luciferease reporter

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pGL3_ARE or pGL3_mARE, 0.02 µg of the *renilla l*uciferase control reporter pRL-TK with or without cotransfection with 0.2 µg expression vectors using Lipofectamine™ 2000 (Invitrogen) as transfection carrier. Treatments with bile acids were then carried out for another 16-18 h in all experiments, unless otherwise stated. Conjugated bile acid treatments (GCA and GCDCA) were done on NTCP- transfected HepG2. Luciferase activities of the cell extracts were determined with the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity (RLU) was calculated from *firefly* luciferase values normalized to those of the control *Renilla* luciferase, and expressed as ratios to vehicle-treated empty pGL3 promoter construct and, if any, cotransfected expression vector. All experiments were done in triplicate and repeated at least twice. For overexpression studies, Hepa1c1c7 cells at 50% confluence in T25 flasks were transfected with 3 µg of Nrf2 or dominant negative Nrf2 expression vector for 24 h, followed by treatments with bile acids for another 20-22 h.

Quantitative Chromatin Immunoprecipitation (ChIP). The assay was performed using the ChIP assay kit (Upstate, Billerica, MA) with slight modifications. After 6 h of treatments, chromatin protein-DNA of HepG2 cells was fixed (cross-linked) in neutral-buffered 1% formaldehyde at room temperature for 10 min. Further fixation was stopped by 125 mM glycine buffer. The DNA was sheared by sonication on ice into fragments of ~ 500 bp in size. An aliquot (1/4) of sample supernatant was saved as input DNA for later PCR analysis. After pre-clearing with protein A agarose beads, supernatants were incubated with a ChIP-graded anti-Nrf2 antibody (1:250; Santa Cruz) in rotation at 4 ^{0}C overnight. To control for nonspecific binding of antibody used, an equal amount of the host antibody against an irrelevant protein (rabbit polyclonal anti-CYP1A1) from the same manufacturer (Santa Cruz) was included in a separate batch of control supernatants and followed through the remaining protocols. Antibody-chromatin complexes were collected by salmon sperm DNA/ protein A beads. DNA was released from crosslinked complexes with proteinase K at 65 ^{0}C for 4 h followed by 72 ^{0}C for 10 min. DNA was then extracted and eluted with 120 µL Tris (pH 8.0) buffer using the DNeasy Kit (Qiagen) and the contaminant RNA was cleaved with RNase A (Invitrogen). For detection of the ARE of GCLM (-56/-66)(Erickson *et al.*, 2002) and of GCLC (-

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3604/-3614)(Mulcahy *et al.*, 1997) by real-time PCR, the primer sets and Taqman probe (5'-Fam, 3'-Tamra) were designed by PrimerQuest software (IDT) which amplify 5'-region exactly on the core ARE. The primers for detecting GCLM's ARE were: sense, 5'-CGCGGGATGAGTAACGGT-3'; antisense, 5'-GGGAGAGCTGATTCCAAACTGA-3'; probe, 5'-ACGAAGCACTTTCTCGGCTACGAT-3' which amplify a 79 bp product (-33/-112). For probing the ARE of GCLC, the primers used were: sense, 5'-GGACTGAGACTTTGCCCTAAGAAG-3'; antisense, 5'-GCGCAGTTGTTGTGATACAG-3'; probe, 5'-CGCACAAAGCGCTGAGTCAC-3' which amplify a 160 bp product (-3479/-3609). Quantitation of NRF2 bound to these AREs after the treatments was carried out on 5% of DNA eluates with qPCR analysis similar to that for the mRNA, except that the Ct value of amplicon from each sample's input DNA was used as normalization control as described (Beresford & Boss, 2001).

Cytotoxicity, Necrosis, and Apoptosis. For cytotoxicity analysis, a nontoxic assay, namely Alamar Blue™ (Biosource, Nivelles, Belgium), was used. This assay uses a fluorometric indicator to measure the chemical reduction of cell medium which correlates directly to the metabolic activity of viable cells. The working assay medium (10% v/v Alamar Blue in α-MEM, 2% FBS, 1% penicillin/streptomycin, 37 ^{0}C) was first incubated with cells seeded on 24-well culture plate prior to treatment to obtain baseline/pretreatment values. The measurement was made at excitation/emission/cut-off λ = 540/590/570 nm after one hour of incubation with the assay medium at 37 ^{0}C. Immediately after the measurement, the cells were rinsed with pre-warmed PBS followed by the treatments. At various time points, treatment media were replaced with fresh assay media to allow for a continuous monitoring of cell viability. The fluorescent unit of each treatment and control was expressed as percent change relative to individual baseline/ pretreatment value.

To determine necrosis, cellular release of lactate dehydrogenase (LDH) into treatment media was measured with a LDH detection kit (Roche Applied Science, Indianapolis, IN). To control for cell mass and spontaneous release of LDH by viable cells into media, the ratio of LDH activity in the medium to the cells (cell lysate) was determined and then subtracted from those of the vehicle-treated

controls. The measurement was made colorimetrically at λ = 490 nm. The intra-assay variability (% CV) of duplicate determinations was 2.2.

To assess apoptosis, the cellular caspases activity was measured using the rhodamine 110-conjugated substrate Asp-Glu-Val-Asp (Z-DEVD-R110)(Invitrogen-Molecular Probes, Carlsbad, CA). Although classically known to detect caspase-3 activity, recent analysis by the manufacturer showed that this substrate is also a target of multiple caspases such as 6, 7, 8 and 10. The caspases activity of cell lysate was quantitated at excitation/emission λ = 496/520 nm, and normalized to individual protein concentration measured by Bio-Rad Protein Assay.

Total Glutathione (GSH) Quantitation. Cellular GSH was quantitated with a GSH assay kit (Cayman Chemical, Ann Arbor, MI) based on an established GSH recycling enzymatic method (Tietze, 1969). After 24 h treatment with bile acids, HepG2 cells were lyzed in ice-cold MES buffer following a quick freeze-thaw cycle and deproteinized by 0.5 g/mL metaphosphoric acid. An aliquot of each sample was saved before deproteinization for determining protein content. The total GSH of deproteinized cell supernatants was measured against a GSSG standard curve according to the manufacturer's instruction. The measurement unit was expressed as nmol/mg protein/min. The intra-assay variability (%CV) of duplicate determinations for all samples repeated in four experiments was 3.1.

Statistical Analysis. Statistical tests were conducted using SigmaStat 3.1 (San Jose, CA) or SPSS10.1 (Chicago, IL). Normality and equal variance tests were first carried out to guide subsequent statistical analyses. Multiple group comparisons were carried out by one-way ANOVA (parametric) or one-way ANOVA on ranks (non-parametric). Once statistical significance was attained ($p<0.05$), the Dunnet's (parametric) or Dunn's (non-parametric) test comparing between treatment and control groups was initiated. Comparisons between two groups on single variable were accomplished by Student's independent t-test (parametric) or Mann-Whitney U test (non-parametric). Difference with $p<0.05$ was considered statistically significant.

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Results

Induction of Nrf2 genes by bile acids in liver and intestinal cells. A dose- response increase in mRNA of GCLM and GCLC following LCA and/or CDCA treatment was noted for HepG2 and C2bbe1 cells with a significant >4-fold induction at ≥50 μM LCA and ≥100 μM CDCA (Fig.1a). Peak inductions of GCL subunit genes occurred at 50-75 μM LCA and 100-150 μM CDCA. Increased bile acid concentrations, i.e., LCA ≥ 100 μM or CDCA ≥ 250 μM, resulted in markedly cell death and reduced inductions of GCL genes at 24 h of treatment (not shown). Significantly higher GCL gene transcripts, although at a lower magnitude (2-4 fold induction), were also noticed for the primary human hepatocytes treated with both bile acids (Fig. 1a). So were similar treatments given to the mouse hepatoma Hepa1c1c7 (not shown). In C2bbe1 cells, CDCA treatment (100 μM) caused a modest increase in GCL genes (~2 folds), whereas treatment with deoxycholic acid (DCA) (≥ 100 μM), a secondary bile acid often associated with toxicity and carcinogenesis in colonic cells, resulted in comparable GCL inductions to those of LCA treatment (not shown).

Furthermore, mRNA of Nrf2 and a panel of known Nrf2 target genes such as NAD(P)H quinone oxidoreductase 1 (NQO1), thioredoxin reductase 1 (TRx1), ferritin light subunit (FRL), and heme oxygenase I (HO1) were also simultaneously induced ≥2-fold by bile acids in all test cells (Fig. 1b). Particularly, TRx1, an important seleno-enzyme in cellular thiol and redox maintenance, was increased >4 folds in HepG2 and C2bbe1. Note that glutathione s-transferase P1 (GSTP1), which was induced by bile acids in HepG2 and C2bbe1, was not evident in primary hepatocytes (Fig. 1b). Instead, another GST subtype, GSTA1, was increased by bile acids to ~2 folds in primary hepatocytes (not shown). This disparity suggests possible cell type specificity in regulation of GSTs by bile acids.

Increased protein levels corresponding to mRNA induction were also noted (Fig. 1c). The apoptosis marker (caspases activity) and cell viability analyses showed that a mild toxicity began to occur in HepG2 cells at 60 – 80 μM of LCA treatment, followed by a precipitous increase in cell death and caspase activity at >80 μM (Fig.1d). Notably, the induction of GCL subunits and other antioxidative genes peaked in the range of LCA (60 – 80 μM) during which HepG2 began to

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experience mild toxicity. These findings suggest that induction of the cytoprotective genes may represent an adaptive cell defense mechanism against LCA toxicity.

In-vivo activation of Nrf2 target genes. Acute administration (7-8 h) of LCA to mice at a dose known to induce cholestatic liver injury (Staudinger *et al.*, 2001) resulted in Nrf2 accumulation in the nuclei, a signature event of Nrf2 activation (Fig.2a). This phenomenon coincided with significant inductions of Nrf2 target genes (Fig. 2b; top panel) found to be increased in the in-vitro studies. Of note, the increase of TRx1 transcripts rose to ~50 folds at acute exposure to LCA, implying a possible critical role of this enzyme in early toxicity of LCA. At this shorter exposure to LCA, however, analysis of serum liver function and cholestatic markers (ALT, AST, GGT and TBL) as well as liver histology did not indicate liver dysfunction or pathological changes (not shown).

With prolonged LCA treatment during which symptomatic liver injury (elevated ALT, AST and TBL, and liver necrosis in histological analysis; not shown) already occurred, induction of Nrf2 target genes, such as GCL subunit gene transcripts, was found to sustain compared with those treated acutely with LCA (Fig. 2b; bottom panel). Nqo1 was increased with prolonged treatment, whereas TRx1 induction was subdued. Consistent with the observations from primary human hepatocytes, α class of mouse Gst (Gsta1/2), rather than Gstp1, was found to be induced by LCA, with ~10-fold induction at 4 d of treatment (Fig. 2b; bottom panel). Treatment with vehicle alone did not differ in mRNA of genes under study when compared with untreated animals (not shown).

Involvement of Nrf2 and activation of Nrf2-ARE transcription machinery. To examine if Nrf2 participated in the preceding gene activations, we silenced Nrf2 of HepG2 and C2bbe1 via siRNA. This resulted in significant reductions of >60% in Nrf2 mRNA and protein without interfering with other homologous Nrf subtypes (Fig. 3a, 3b). Nrf2 silencing significantly decreased the basal levels of known Nrf2 target genes (Fig. 3c, 3d, 3e), an observation similar to those seen in in-vivo Nrf2 knockout mice (Lee *et al.*, 2005). Also, the induction of GCLM, GCLC and other Nrf2 target genes by bile acids in HepG2 (Fig. 3c, 3e) and C2bbe1 (Fig. 3d) has been mitigated. Comparable reduction in inducible expressions of Gclm occurred with transfection of dominant-

negative Nrf2 in Hepa1c1c7 cells, consistent with the enhanced gene induction with Nrf2 overexpression (Fig. 3f). Similar observations were noted for other Nrf2 target genes such as Gclc and Nqo1 (not shown).

To verify that there was an activation of Nrf2-ARE transcription machinery with exposure to toxic bile acids, we extracted the nuclear proteins of bile-acid treated HepG2 over different time points across 24 h. Translocation of cytosolic Nrf2 to nucleus represents the prerequisite event of receptor activation. Nrf2 started to be enriched in cell nuclei within 1-3 h of bile acid treatments and sustained through 24 h, with CDCA-treated cells showed reduced Nrf2 translocation events with longer time of exposure (24 h) (Fig. 4a). Further, various bile acids were found to increase the activity of an ARE-reporter assay in a dose-dependent manner, suggesting that these bile acids were capable of inducing Nrf2 transactivation (Fig. 4b). The magnitude of luciferase activity of the highest concentration of test bile acids was compatible to those of treatments with antioxidants tBHQ (100 μM) and α-lipoic acid (200 μM) (not shown), denoting that bile acids are equally potent Nrf2 activators. Note that there was an ~8-fold increase in reporter activity with vehicle DMSO treatment compared with that of the empty vector harboring only the SV40 promoter. This suggests the existence of a strong constitutive Nrf2-ARE transactivational activity in HepG2 cells, an observation in line with the persistent oxidative stress observed in many cancerous cell lines (Brown & Bicknell, 2001). HepG2 cells are deficient in conjugated bile acid transporters such as NTCP which leads to its resistance to conjugated bile acid-induced oxidative stress (Kullak-Ublick *et al.*, 1996). Transfection of NTCP expression vector hence restores, to some degree, its sensitiveness. In this study, we found that GCDCA, a known cholestatic conjugated bile acid, significantly induced the ARE reporter. This suggests that activation of Nrf2-ARE may be crucial to counteract the toxicity of GCDCA as reported previously (Dent *et al.*, 2005). The potency of bile acids in activating this reporter based upon molarities was: LCA > CDCA ≈ DCA > GCDCA ≥ UDCA > CA > GCA. This order is in consensus with the toxicity profile of these bile acids, particularly in terms of their ability to produce oxidative stress (Krahenbuhl *et al.*, 1994). Overexpression of Nrf2 further enhanced the

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reporter activity by bile acids, whereas coexpression of dominant-negative Nrf2 attenuated the activity, and a mutant ARE construct was completely uninducible by bile acids (Fig. 4c). Using the quantitative ChIP assay, we found an increased Nrf2 occupancy to the AREs of both GCL subunits in the native cell context upon 6 h treatment with bile acids (Fig. 4d). Taken together, our data suggest that activation of the Nrf2-ARE machinery underlies induction of Nrf2-target genes by toxicological concentrations of bile acids.

Protective role of Nrf2 in bile acid toxicity. To directly investigate the role of Nrf2 in protection against toxic bile acids, we first silenced Nrf2 of HepG2 via RNAi upon which the cells were subjected to toxic LCA challenges. Nrf2 knockdown increased cell susceptibility to toxic LCA with a significantly decreased cell viability starting 4 h of treatment (Fig. 5a). Without LCA challenge, Nrf2-knockdown cells did not differ in cell viability from those treated with siRNA control (siCtr) (not shown). Significant protective effects of Nrf2 against LCA toxicity was also observed in C2bbe1 cells, and in HepG2 with $\geq$ 300 μM CDCA (not shown). To investigate which route of cell death was particularly involved in Nrf2's protection, established markers of necrosis and apoptosis were examined. LCA at 90 μM was used as at this dose both apoptosis and necrosis were found to simultaneously occur. Necrotic event, as determined by LDH released into the culture media, remained constantly higher in Nrf2 knockdown cells than those of siCtr starting from 2 h of LCA treatment (Fig. 5b). Nrf2 silencing alone did not affect the cellular release of LDH (not shown). Similarly, in the assessment of apoptosis, Nrf2-knockdown cells exhibited much higher and prolonged elevation of caspases activity than were siRNA control-treated cells upon LCA treatment (Fig. 5c). Silencing of Nrf2 alone did not result in increased basal caspases activity.

The role of GSH in resisting LCA toxicity. The increase in GCLM and GCLC, the rate-limiting enzyme in GSH biosynthesis, observed in earlier experiments after LCA (75 μM) or CDCA (100 μM) treatment was accompanied by a significant increase by >4-fold in cellular GSH levels at 24 h (Fig. 6a). This increase was comparable to treatment with 200 μM α-lipoic acid, a GSH inducer. To determine whether the induced cellular GSH is a protective mechanism against toxic bile acid, we

cotreated HepG2 with a toxic dose of LCA and a GSH biosynthesis blocker, buthionine sulfoximine (BSO) which inhibits the activity of GCL subunits and blocks GSH biosynthesis. BSO treatment together with toxic LCA decreased cell resistance toward LCA exposure with more apparent effects in late treatment (~24 h) (Fig. 6b). Furthermore, depletion of cellular GSH by pretreatment with BSO prior to LCA challenge markedly lifted cell resistance with a drastic drop in cell viability within 4 h of treatment. Conversely, toxic LCA challenge in the presence of an antioxidant and GSH precursor N-acetyl-L-cysteine (NAC) was found to alleviate the toxicity. This suggests that the basal as well as inducible GSH are important determinants of cellular resistance to LCA. Consistent with these findings, NAC cotreatment significantly reduced the oxidative stress-responsive ARE-reporter activity by LCA, indicating an antioxidative effect (Fig. 6C). BSO cotreatment, on the other hand, further increased the reporter activity, consistent with a heightened oxidative stress (Fig. 6c).

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Discussion

The discovery of bile acids as key signaling molecules in the enterohepatic circulation system reveals a critical role of hepatic and intestinal xenobiotic nuclear receptors in the metabolism and detoxification of bile acids (Chawla *et al.*, 2000). Particularly, the cytotoxic hydrophobic bile acids, CDCA and LCA, have been shown to be ligands and potent inducers of these receptors. LCA, at physiological and non-toxicological concentrations (5-30 μM) can activate FXR (Makishima *et al.*, 1999; Makishima *et al.*, 2002) and VDR (Makishima *et al.*, 2002), indicating their crucial role in physiological handling of this bile acid. The major detoxification routes of LCA, i.e., sulfation by sulfotransferase 2A (SULT2A) and 7α-hydroxylation by CYP3As, are coordinated by VDR (Makishima *et al.*, 2002; Echchgadda *et al.*, 2004). FXR, which activates the hepatic bile salt export pump BSEP (Ananthanarayanan *et al.*, 2001) and downregulates the bile-synthesizing enzyme CYP7A1 (Makishima *et al.*, 1999), works to prevent intracellular accumulation of bile acids.

Interestingly, at higher and toxicological concentrations of LCA (≥ 50 μM) and CDCA (≥ 100 μM) which potentially cause cell injury, PXR (Staudinger *et al.*, 2001; Makishima *et al.*, 2002) and Nrf2, as shown in this study, are found to be activated. The activation of PXR and Nrf2 induces the major hydroxylation enzymes CYP3As and antioxidative genes (Eloranta & Kullak-Ublick, 2005; Kensler *et al.*, 2006), which may represent an important adaptive mechanism of cellular defense against toxic bile acids. Furthermore, we observed that induction of multiple bile salt/conjugate efflux transporters such as ATP-binding cassette (ABC) transporters ABCC2, ABCC3 and ABCG2 by bile acids is dependent on Nrf2 (Tan *et al.*, unpublished data[Tan1]). Hence, the collective induction of cytoprotective genes by Nrf2 and PXR appears to set off a second line of protection against possible progression of bile acid toxicity toward irreversible cell death.

In this study, we showed for the first time that many bile acids, more potently LCA, CDCA and DCA, are capable of activating redox-sensitive Nrf2. We also provided in-vivo evidence that LCA is able to activate Nrf2, inducing similar target genes observed in in-vitro studies. The induction of Nrf2 target genes by LCA in-vivo was found to precede and sustain through biochemically and

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histologically overt liver injury, suggesting that the collective induction of these antioxidative genes may be an integral part of cell defense against bile acid toxicity and hepatic injury. Previous studies have reported an increased intracellular production of detrimental hydroperoxides in isolated rat hepatocytes with hydrophobic bile acid exposure (Sokol *et al.*, 1995), an observation in consensus with the increased oxidative stress byproducts in the liver of patients with cholestasis (Vendemiale *et al.*, 2002). Since Nrf2 activation is indicative of cellular antioxidative response, our study provides molecular evidence linking mechanism of bile acid toxicity to oxidative stress.

We further showed that induction of hepatic GCL subunits via Nrf2 which provokes GSH biosynthesis can increase hepatocyte resistance and survival during excessive bile acid exposure. The essential role of GSH in hepatic protection against injury and oxidative xenobiotic insults has been well exemplified (Huang *et al.*, 2001; Glosli *et al.*, 2002). In agreement, in-vivo knockout of Nrf2 enhances sensitivity of death receptor-induced hepatic apoptosis as a result of decreased GSH levels (Morito *et al.*, 2003). GSH is also known to protect against mitochondrial injury, a major mechanism of bile acid toxicity (Palmeira & Rolo, 2004). A fraction of cytosolic GSH which becomes mitochondrial GSH is crucial in the defense of oxidant-induced mitochondrial-mediated cell death (Fernandez-Checa & Kaplowitz, 2005). Additionally, Nrf2 activation has been shown to protect mitochondria by preventing inhibition of mitochondrial complex II upon exposure to oxidative neurotoxins (Calkins *et al.*, 2005). In intestinal mucosa, cellular GSH has an essential role in maintaining epithelial integrity, transport activity, and metabolism of and susceptibility to luminal toxins (Aw, 2005). Overall, our studies, coupled with supportive evidence from recent literature, suggest that protection conferred by hepatic and intestinal Nrf2 against bile acid-induced oxidative stress is, at least partly, by increasing GSH levels.

The simultaneous induction of other Nrf2 target genes may work in concert with GCL subunits to combat bile acid-induced oxidative stress while facilitating adaptive responses. Of particular importance is TRx1, an enzyme engaged in NADPH-dependent catalysis of various redox proteins (Rundlof & Arner, 2004). It has been shown to act as a key adaptation-promoting mediator

for prior exposure to 4-hydroxynonenal, a reactive lipid peroxidation-derived molecule, in inducing cellular tolerance to future oxidative stress attack (Chen *et al.*, 2005). Indeed, intermediate cellular stress has recently been proposed to provide an adaptation advantage by invoking enhanced cellular survival/tolerance mechanisms (Schoemaker *et al.*, 2003; Chen *et al.*, 2005). Activation of NF-κB as well as Nrf2 has been shown to play an important role in this adaptation process. The drastic induction of TRx1 observed in mice upon acute exposure to toxic LCA in this study may indicate a critical role of this enzyme in adaptation process against LCA toxicity. To address whether and how this process is taking place, future studies are needed.

The precise mechanisms by which toxic bile acids activate Nrf2 remain a subject of future studies. Enormous production of ROS from mitochondrial stress has long been accounted for the main source of oxidative stress induced by bile acids (Palmeira & Rolo, 2004). Insurgence of these ROS potentially targets the cysteine oxidative-sensors of Keap1, an actin-anchored cytosolic sequester that facilitates Nrf2 degradation by ubiquitin-proteosome pathway, which leads to liberation and activation of Nrf2 (Kensler *et al.*, 2006). Also, subsets of both conjugated and unconjugated bile acids have been shown to activate multiple kinase signaling pathways such as PKC, ERK1/2 MAPK, p38 MAPK, JNK, and/or PI-3/AKT (Dent *et al.*, 2005; Debruyne *et al.*, 2002). These signaling pathways have been shown as well to influence the stability of Nrf2-Keap1 complex, and post-transcriptionally regulate the Nrf2 target genes (Kensler *et al.*, 2006).

In summary, we characterized a molecular cell defense event associated with bile acid-provoked oxidative stress. Exposure to cytotoxic bile acids in the liver and intestinal cells was shown here to cause Nrf2 activation, thereby upregulating a battery of cytoprotective genes, particularly GCL subunits, to enhance cell survival at the emergence of oxidative stress.

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Acknowledgments

We thank Dr. Jawed Alam for providing expression vector plasmids, Dr. Patricia Harper for providing Hepa1c1c7 cells, and Christopher Tierney (Dharmacon) for technical assistance in RNAi studies.

Molecular Pharmacology Fast Forward. Published on August 27, 2007 as DOI: 10.1124/mol.107.039370
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Footnotes

a) This work is supported by Canadian Institute for Health Research Grant (MT13747 to S.I.); K.P.T was supported by the RESTRACOMP studentship, Research Institute, Hospital for Sick Children and University of Toronto Fellowship.

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Molecular Pharmacology Fast Forward. Published on August 27, 2007 as DOI: 10.1124/mol.107.039370
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Legends of Figures

Figure 1. Induction of Nrf2 target genes by bile acids. a, mRNA levels of GCLM (white bar) and GCLC (black bar) in HepG2 and C2bbe1 cells (left panel) after 24 h treatment with increasing doses of LCA or CDCA: [LCA] = 6.25, 12.5, 25, 50, 75 μM; [CDCA] = 25, 50, 75, 100, 150 μM. Right panel: human primary hepatocytes (passages# 4-6) were treated with 75 μM LCA or 100 μM CDCA for 24 h. Y-axis: fold change vs. vehicle-treated controls. *Significantly different ($p<0.05$) from vehicle-treated control by one-way ANOVA followed by posthoc test for HepG2 and C2bbel, or ** $p< 0.01$ by t-test for primary hepatocyte. Mean and SEM (n=3-6). **b,** mRNA levels of other known Nrf2 target genes after 24 h treatment with bile acids in HepG2, C2bbe1 and primary hepatocytes; [LCA] = 75 μM; [CDCA] = 100 μM. Mean and SEM (n=3-6).**c,** Representative immunoblots of protein lysate (10 μg for HepG2, 30 μg for C2bbe1) probed for Nrf2 target genes after 24 h treatment with bile acids. [LCA] = 70 μM; [CDCA] = 100 μM. **d**, Cell viability (by Alamar Blue^{TM}) and apoptotic marker (caspases activity) measured across increasing concentrations of LCA treatment in HepG2. Viability was measured at 24 h while caspases activity was 6 h of LCA treatment. Note that the induction of GCL genes peaked at 60-80 μM of LCA (shown by closed inverted triangles) during which mild cellular toxicity began to occur. Representative results from 4 determinations are shown.

Figure 2. Activation of Nrf2 in mice treated with LCA. a, Representative immunoblots of nuclear fractions (30 μg) probed against Nrf2 in the liver of mice after 7-8 h treatment with cholestatic LCA (125 mg/kg body wt.). Lamin B was used as loading control for nuclear protein, whereas β-actin was probed to show unintentional contamination of cytosolic proteins in nuclear fraction preparation. Note that the increased nuclear Nrf2 cannot be explained by inclusion of contaminant cytosolic proteins. **b,** mRNA levels of Nrf2 target genes upon LCA treatment for 7-8 h (top panel) or for 4 d (bottom panel) in the liver of mice. Because there were differences in the basal gene expression of Nrf2 target genes between sexes, comparisons of all target genes between treated and untreated groups were adjusted for sex. Induction of

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antioxidative genes by LCA, however, occurred to both sexes. Significantly different from vehicle-treated controls by t-test. * $p<0.05$; ** $p<0.01$. Mean and SEM (n=5-9 for 7-8 h treatment; n=3 for 4 d treatment).

Figure 3. Involvement of Nrf2 in induction of glutamate cysteine ligase subunits by bile acids. a, mRNA levels of all Nrf subtypes after 72 h treatment with siRNA. Y-axis: fold change vs. vehicle-treated cells transfected with control siRNA (siCtr). *Significant difference ($p<0.001$) between siRNA groups by t-test. Mean and SEM (n=3-5). **b,** Representative immunoblots (20 µg protein lysate) of HepG2 after 48 h treatment with siRNA against Nrf2. **c,** Basal (treated with vehicle DMSO) and inducible (treated with 70 µM LCA or 100 µM CDCA) gene transcripts of GCLM and GCLC in HepG2 (mean and SEM: n=3-5); and **d,** basal and inducible levels of GCLM and GCLC gene transcripts in C2bbe1 treated with vehicle or 70 µM LCA. *Significant difference ($p<0.01$) between siCtr and siNrf2 with or without treatment by t-test. Mean and SEM (n=3-5). **e,** Basal and inducible expressions of other Nrf2 target genes in HepG2. Mean ± SEM. **f,** mRNA levels of GCLM induced by 70 µM LCA or 100 µM LCA in Hepa1c1c7 transfected with empty vector (pEF), Nrf2 (pEF_Nrf2) or dominant negative Nrf2 (pEF_DNrf2) expression vector. Y-axis: fold change vs. vehicle-treated cells transfected with empty vector pEF. *Significantly different ($p<0.05$) from LCA-treated cells transfected with pEF control by t-test. Mean and SEM (n=4).

Figure 4. Bile acids activate Nrf2 transcription machinery. a, Representative immunoblots of nuclear fraction (10 µg) extracted from HepG2 treated with bile acids (LCA, 70 µM; CDCA, 100 µM) at indicated time-points over 24 h. Lamin B was used as equal loading control for nuclear protein whereas β-actin was probed to show possible contaminant cytosolic proteins in nuclear fraction preparation. **b,** ARE-reporter (luciferase) activity of HepG2 treated with increasing doses of various bile acids for 16-18 h. Abbreviations (doses): LCA (50, 70, 90 µM); CDCA (50, 100, 150 µM) CA, cholic acid (150, 200, 400 µM); DCA, deoxycholic acid (50, 100, 150 µM); UDCA , ursodeoxycholic acid (50, 100, 200 µM); GCA, glycocholic acid (200, 400, 800 µM); GCDCA, glycochenodeoxycholic acid (100, 200, 400 µM). # GCA

and GCDCA were tested in NTCP-transfected HepG2 for 6 h. Y-axis: fold change in the ratio of luciferase activity (relative luciferase unit)(please see methods and materials for detail) from those transfected with basic pGL3 promoter construct and treated with vehicle DMSO. *Significantly different ($p<0.05$) from DMSO-treated pGL3_ARE by one-way ANOVA followed by posthoc test. Mean and SEM (n= 2-4) **c,** ARE-reporter (pGL3_ARE) activity with coexpression of Nrf2 or dominant-negative Nrf2, and mutant ARE-reporter (pGL3_mARE) activity in HepG2 treated with bile acids (LCA = 70 μM; CDCA = 100 μM). Y-axix: fold change in the ratio of luciferase activity (relative luciferase unit) from those transfected with basic pGL3 promoter construct and/or respective expression vector, and treated with DMSO. *Significant difference ($p<0.05$) between vehicle control and bile acids by one-way ANOVA followed by posthoc test. Mean and SEM (n=3-6). **d,** ChIP analysis examining Nrf2 occupancy to AREs of both GCLM and GCLC genes upon 6 h treatment with bile acids (70 μM LCA; 100 μM CDCA) in HepG2. BHQ (200 μM), known to transcriptionally activate GCLM and GCLC, was included as positive control. Negligible detection from samples incubated with host IgG (anti-CYP1A1) ruled out contribution of non-specific binding from antibody. *Significantly different ($p<0.05$) from controls (t=0) and vehicle treatment by t-test. Mean and SEM of triplicate determinations of representative experiments.

Figure 5. Nrf2 is a cellular protector against LCA-induced toxicity. a, Cell viability of HepPG2 after knockdown of Nrf2 via siRNA prior to treatment with toxic LCA (100 and 120 μM). Viability was measured at indicated time-points over 24 h. Fluorescent values of each LCA-treated siRNA group was first subtracted by the average values of individual siRNA group treated with vehicle DMSO, and expressed as percent change to baseline/pretreatment values. *Significant difference ($p<0.01$) between siCtr and siNrf2 groups with t-test. Representative results; mean ± SEM of 4 determinations. **b,** Analysis of LDH release ratio, a marker of necrotic event or cell injury, upon LCA challenge (90 μM) in HepG2 after Nrf2 silencing. Values of LCA treatment were subtracted by the average values of vehicle treatment for each siRNA group. Representative results were presented; mean of duplicate determinations. **c,**

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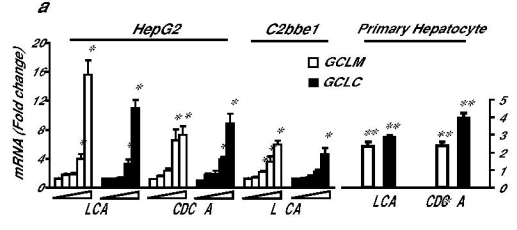
Analysis of caspases activity upon triggered by LCA toxicity (90 μM) in HepG2 knockdown of Nrf2. Representative results of duplicate determinations were shown.

Figure 6. Increased GSH production is a cellular protective mechanism against bile acid toxicity. a, Cellular GSH levels of HepG2 after 24 h treatment with 75 μM LCA, 100 μM CDCA or 200μ lipoic acid (LA; positive control). Buthionine sulfoximine (BSO; 60 μM), a GSH synthesis blocker, was used as negative control. *Significantly different ($p<0.05$) from DMSO control by one-way ANOVA followed by posthoc test. Mean and SEM (n=4). **b,** Cell viability of HepG2 treated with toxic LCA (100 μM) together with 0.4 mM NAC, or 60 μM BSO, or with overnight pretreatment with 60 μM BSO (preBSO). Fluorescent units were first subtracted by those of respective treatment control (i.e., DMSO, NAC or BSO alone), and expressed as percent change to individual baseline/pretreatment values. *Significantly different from LCA treatment by one-way ANOVA followed by posthoc test ($p<0.05$). Mean ± SEM of 3 independent experiments with 4 determinations. **c,** ARE-reporter activity in HepG2 treated with LCA with or without 0.4 mM NAC or 60 μM BSO. Tert-butylhydroperoxide (tBHP, 100 μM), a peroxide radical generator, was used to show that increased cellular oxidative stress led to increased ARE reporter activity. Y-axis: fold change in the ratio of luciferase activity (relative luciferase unit) from those transfected with basic pGL3 promoter construct and treated with vehicle DMSO. *Significant difference ($p<0.05$). Chemical treatments were for 8 h. Mean and SEM (n=3-4).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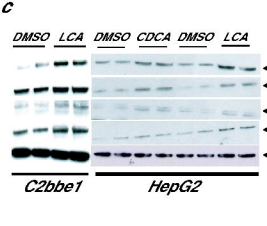
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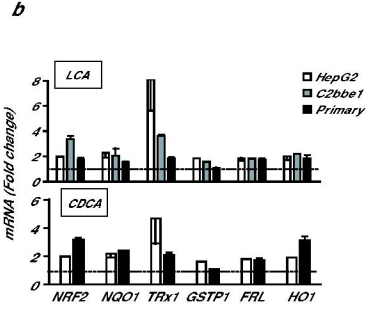
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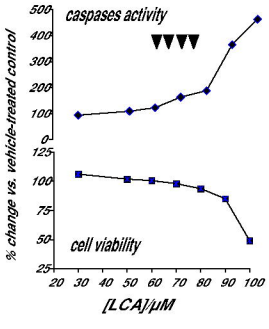
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[LCA]/μM

Figure 1

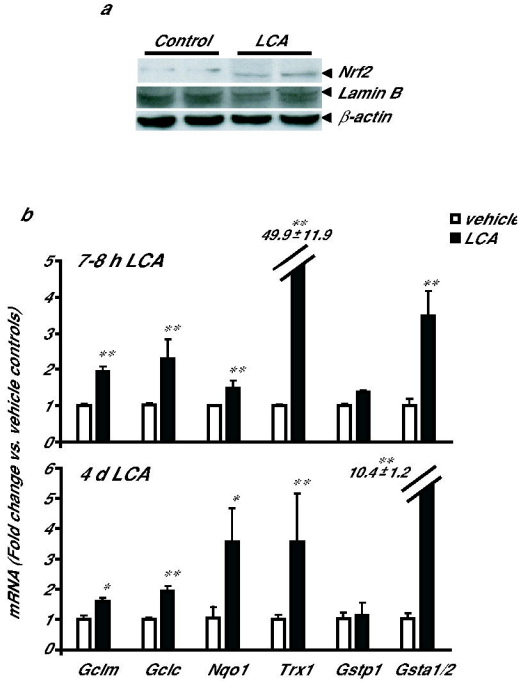
a
Control
LCA
Nrf2
Lamin B
β-actin
b
vehicle
LCA
7-8 h LCA
49.9±11.9
4 d LCA
10.4±1.2
mRNA (Fold change vs. vehicle controls)
Gclm
Gclc
Nqo1
Trx1
Gstp1
Gsta1/2

Figure 2

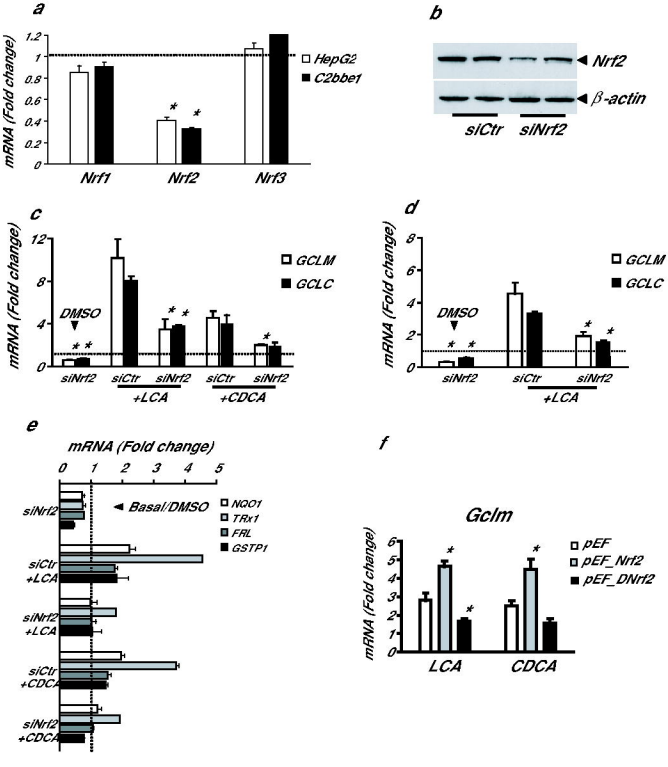
a
mRNA (Fold change)
HepG2
C2bbe1
Nrf1
Nrf2
Nrf3
b
Nrf2
β-actin
siCtr
siNrf2
c
mRNA (Fold change)
GCLM
GCLC
DMSO
siNrf2
siCtr
siNrf2
+LCA
siCtr
siNrf2
+CDCA
d
mRNA (Fold change)
GCLM
GCLC
DMSO
siNrf2
siCtr
siNrf2
+LCA
e
mRNA (Fold change)
Basal/DMSO
NQO1
TRx1
FRL
GSTP1
siNrf2
siCtr +LCA
siNrf2 +LCA
siCtr +CDCA
siNrf2 +CDCA
f
Gclm
mRNA (Fold change)
pEF
pEF_Nrf2
pEF_DNrf2
LCA
CDCA

Figure 3

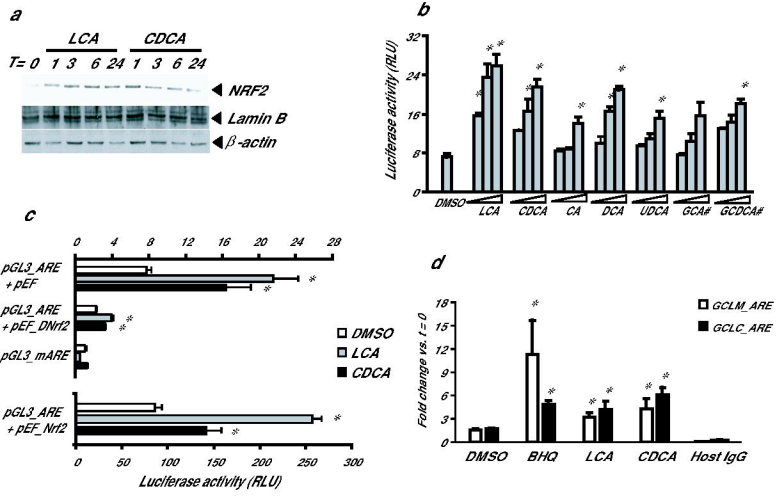
a
LCA
CDCA
T= 0 1 3 6 24 1 3 6 24
NRF2
Lamin B
β-actin
b
Luciferase activity (RLU)
32
24
16
8
0
DMSO
LCA
CDCA
CA
DCA
UDCA
GCA#
GCDCA#
c
0 4 8 12 16 20 24 28
pGL3_ARE + pEF
pGL3_ARE + pEF_DNrf2
pGL3_mARE
pGL3_ARE + pEF_Nrf2
DMSO
LCA
CDCA
0 50 100 150 200 250 300
Luciferase activity (RLU)
d
Fold change vs. t = 0
18
15
12
9
6
3
0
GCLM_ARE
GCLC_ARE
DMSO
BHQ
LCA
CDCA
Host IgG

Figure 4

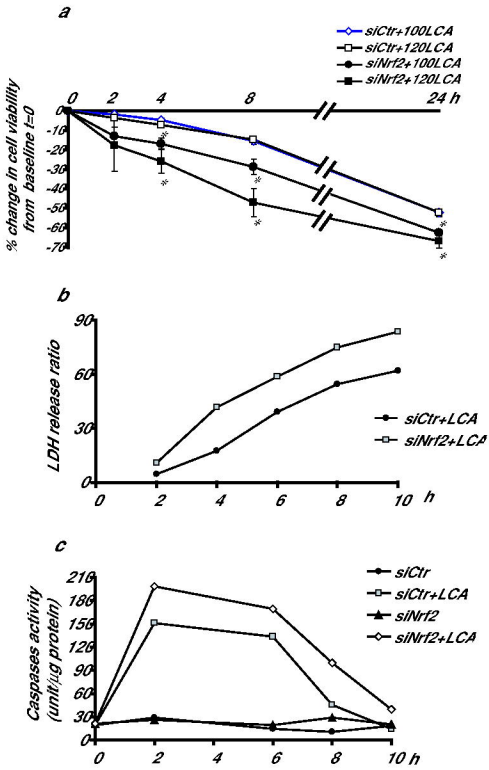
a
siCtr+100LCA
siCtr+120LCA
siNrf2+100LCA
siNrf2+120LCA
% change in cell viability from baseline t=0
0
2
4
8
24 h
b
LDH release ratio
siCtr+LCA
siNrf2+LCA
h
c
Caspases activity (unit/μg protein)
siCtr
siCtr+LCA
siNrf2
siNrf2+LCA
h

Figure 5

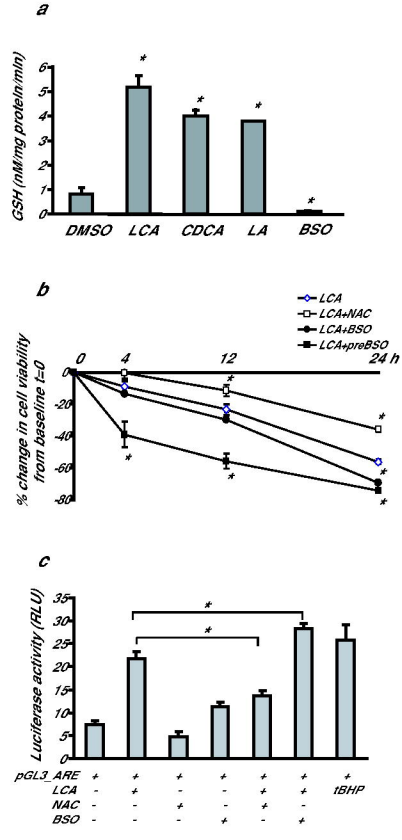
a
GSH (nM/mg protein/min)
DMSO
LCA
CDCA
LA
BSO
b
LCA
LCA+NAC
LCA+BSO
LCA+preBSO
% change in cell viability from baseline t=0
0
4
12
24 h
c
Luciferase activity (RLU)
pGL3_ARE
LCA
NAC
BSO
tBHP

Figure 6